PREFACE

During the past five to ten years, a variety of tools has been developed in the disciplines of both gene engineering, and molecular and structural biology. Some of these advances have permitted scientists not only to identify and characterize genes, but also to target these genes by disruption, thus eliminating their function in living animals, and to determine the biological responses to altered gene products. This has particular significance in endocrine systems, in which feedback mechanisms between the hypothalamus, pituitary, and end organs are critical in normal physiology. Interpretation of the physiological significance, or the site of action of specific molecules in this context, has been difficult prior to transgenic technology. Major advances have occurred specifically in the areas of growth and development, and of reproduction.

Coupled with analysis of naturally occurring mutations in humans, the use of transgenic animals and in vitro systems has recently allowed endocrinologists to understand the importance of specific thyroid hormone receptor isoforms in vivo, the molecular basis for generalized resistance to thyroid hormones via mutations in the nuclear receptor, and mechanisms for suppressing gene transcription. Previously designated "orphan receptors," such as steroidogenic factor-1, were demonstrated to have critical roles in development and reproduction. Other nuclear receptors—including those for thyroid hormone, estrogens, androgens, and progesterone—were shown to bind to coactivator and corepressor proteins that modified their transcriptional activity, and contributed to the cell-specific effects of the hormones. Previous dogma on the independence of steroid and peptide hormone mechanisms of action was shown to be simplistic. In fact, intracellular signaling pathways initiated by peptides modify steroid receptors directly and modulate their activity. These pathways also modify other transcription factors that, alone or in partnership with other proteins, regulate cell-specific patterns of gene expression. The application of transgenic and molecular techniques to the study of reproductive endocrinology illuminated the importance of estrogen in both males and females, the genetic basis for androgen insensitivity, gender-specific roles of the gonadotropins in normal reproduction, and the critical role played by activins, inhibins, and related growth factors.

In view of these tremendous advances, and the ability to draw clinical endocrine correlates from these findings, *Gene Engineering in Endocrinology* was assembled to include contributions from many leaders in these areas. The intent of our book is to place this new information in physiological perspective and to review the most recent work, as well as to indicate the areas of interest and questions that need still to be addressed in future research. The chapters describe studies performed with many types of molecular methods, and the use of animal and cellular model systems to explore the molecular basis of growth, development, and reproduction. Gene manipulation and disruption or "knockout" results are discussed in the context of the impact of specific genes on these physiological systems, and the developmental or physiological time period at which the mutation becomes critical. The molecular studies are compared, when possible, with naturally occurring human and animal gene mutations, in order to compare complete elimination of gene function with an altered gene product.

Gene Engineering in Endocrinology is aimed at a broad spectrum of readers, including those who are currently interested and actively working in molecular endocrinology, and clinical endocrinologists interested in relating molecular mechanisms to clinical endocrinology.

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2 Insulin Action

Molecular Mechanisms and Determinants of Specificity

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and Simeon I. Taylor, MD, PhD

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INTRODUCTION

Insulin is a peptide hormone that plays critical roles in the regulation of growth, differentiation, and metabolism. The physiological importance of insulin is underscored by the fact that the insulin receptor has been evolutionarily conserved and is found in organisms ranging from Drosophila to humans. Furthermore, insulin-dependent diabetes mellitus, a disease characterized by absolute insulin deficiency, was a uniformly fatal condition before the advent of insulin therapy. Like other circulating polypeptide hormones, insulin initiates its biological actions by binding to specific cell-surface receptors. The molecular cloning of the insulin receptor led to the discovery that it belongs to a large family of ligand-activated receptor tyrosine kinases (RTKs) that includes receptors for many other growth factors *(1–5)*. Many of the molecules involved with the transduction of signals from a multitude of RTKs also participate in insulin signaling. One of the central puzzles in the field of signal transduction is understanding how signal specificity is achieved after the interaction of the ligand with its receptor since so many postreceptor events seem to be shared in common by a variety of different RTKs. In this chapter, we briefly review the current understanding of how insulin receptor signaling follows a general paradigm for RTK signal transduction. Particular emphasis is given to signaling pathways related to glucose transport since this is among the most important physiological actions of insulin and is a specialized metabolic function that

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distinguishes the insulin receptor from other RTKs. Finally, we discuss several potential mechanisms for achieving signal specificity that are illustrated by recent studies relevant to insulin signaling.

INSULIN SIGNALING FOLLOWS PARADIGM FOR RTK SIGNAL TRANSDUCTION

As illustrated in Fig. 1, the propagation of information resulting from the binding of insulin to its cell surface receptor follows a general paradigm for RTK signal transduction that ultimately culminates in multiple biological effects, including increased glucose transport, gene and enzyme regulation, and mitogenesis, that are important for the regulation of metabalism and growth.

Ligand Binding and Receptor Dimerization

The first step in initiating signal transduction by an RTK involves the specific binding of a ligand to the extracellular portion of its cognate cell-surface receptor. In the case of monomeric receptors such as the epidermal growth factor (EGF) receptor, ligand binding results in receptor dimerization, a necessary first step in signal transduction *(4,6,7)*. The insulin receptor has a heterotetrameric structure (actually, a dimer of $\alpha\beta$ heterodimers) consisting of two extracellular α-subunits and two transmembrane β-subunits joined by disulfide bonds *(8)*. Thus, even in the absence of ligand, the insulin receptor exists in a dimeric form. The α-subunit of the insulin receptor contains fibronectin III repeats and cysteine-rich domains that are also found in several other RTKs. Insulin binds with high affinity to specific regions of the α-subunit (including the cysteine-rich domain), resulting in a rapid conformational change in the receptor *(9,10)*. In the absence of ligand, the α-subunit of the insulin receptor appears to exert a tonic inhibitory influence on insulin receptor function because insulin receptors that have had the α-subunit removed by trypsin digestion or expression of the cytoplasmic domain of the insulin receptor alone results in constitutive activation of receptor signaling *(11,12)*.

Receptor Autophosphorylation and Activation of Intrinsic Tyrosine Kinase

Ligand binding and receptor dimerization result in activation of the RTK. The kinase region of all RTKs shares substantial homology in both the adenosine triphosphate binding site and the catalytic domain *(4)*. The kinase of one half of the receptor dimer phosphorylates cytoplasmic tyrosine residues on the other half of the receptor dimer. This mutual transphosphorylation event is known as receptor autophosphorylation and results in a large increase in the catalytic activity of the receptor. The β-subunit of the human insulin receptor contains tyrosine residues distal to the catalytic domain at positions 1158, 1162, and 1163 (in the so-called activation loop) that undergo autophosphorylation and are important for enhancing the tyrosine kinase activity of the receptor *(13–15)*.

Tyrosine Phosphorylation of Cellular Substrates and Recruitment of Distal Signaling Molecules

Activation of the RTK leads to tyrosine phosphorylation of cellular substrates that propagate signaling. In addition, receptor autophosphorylation enables the RTK to

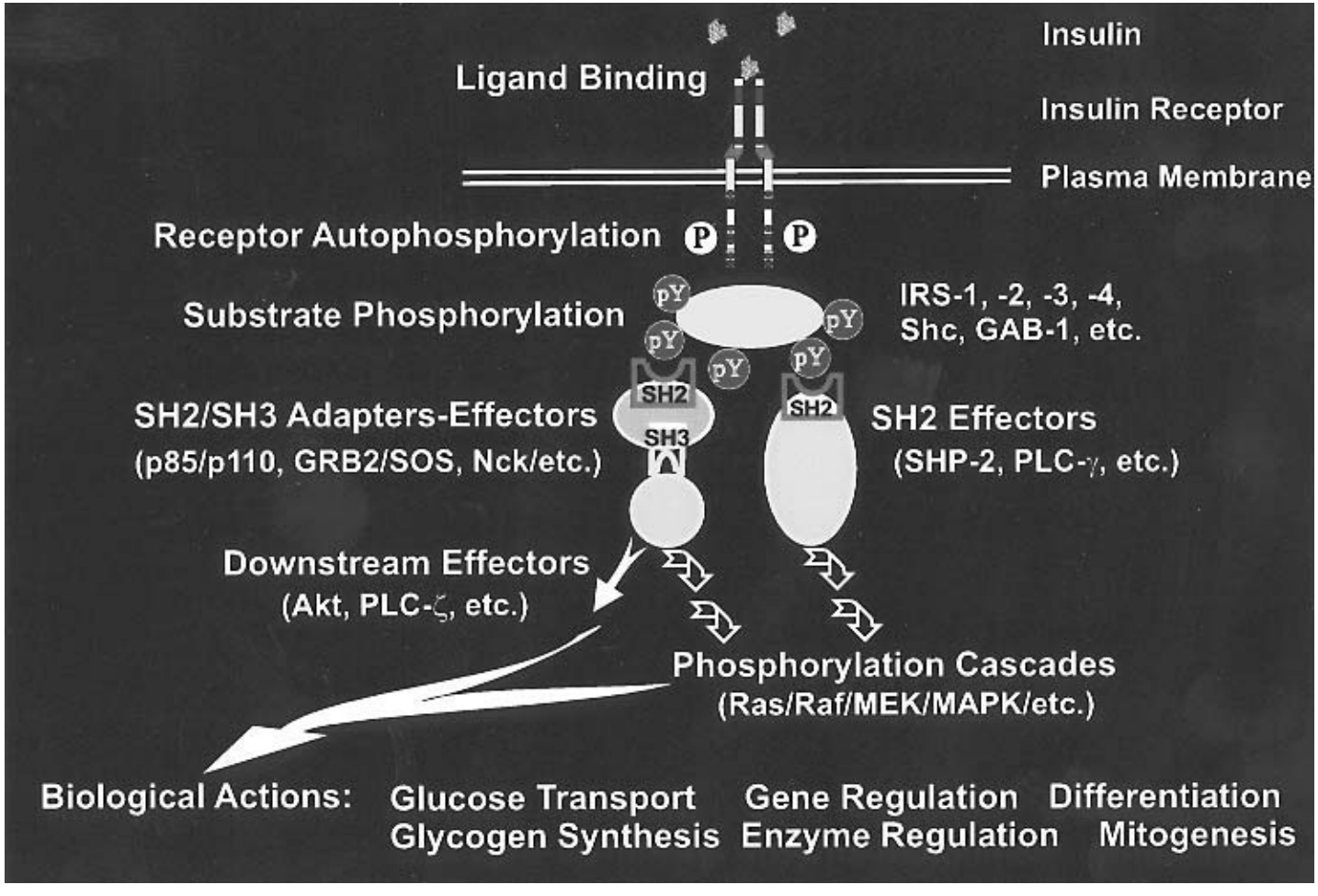

Fig. 1. Insulin signal transduction follows paradigm for RTK signaling.

directly engage signaling molecules via interactions between phosphotyrosine motifs on the receptor and src homology-2 (SH2) domains on downstream molecules. SH2 domains are protein domains of ~100 amino acids that share homology with a noncatalytic region of the src protooncogene product. Many molecules involved with RTK signaling, including src, phosphatidylinositol 3-kinase (PI3K), growth factor receptor-bound protein 2 (GRB-2), SH2-containing phosphatase-2 (SHP-2), GTPase-activating protein (GAP), and phospholipase C-γ (PLC-γ), contain SH2 domains. Motifs defined by the three amino acid residues on the C-terminal side of the phosphotyrosine residue provide specificity for interaction with particular SH2 domains *(16,17)*. In the case of receptors for EGF and platelet-derived growth factor (PDGF), the particular phosphotyrosine sites that engage specific SH2 domains of various signaling molecules have been well mapped *(18,19)*.

Although the autophosphorylated insulin receptor β-subunit is capable of directly interacting with molecules such as PI3K, SHP-2, and GAP *(20–22)*, direct binding of phosphotyrosine motifs on the insulin receptor with SH2 domain-containing molecules does not appear to be the major pathway for insulin signal transduction. Instead, there are substrates of the insulin receptor tyrosine kinase such as insulin receptor substrate-1 (IRS-1), IRS-2, IRS-3, IRS-4, SHC, and GRB-2-associated binder-1 (GAB-1) that provide an interface between the insulin receptor and downstream SH2 domain–containing molecules *(23–29)*. The IRS family of proteins contain a number of conserved regions including a pleckstrin homology (PH) domain and a phosphotyrosine binding domain that are important for the ability of the autophosphorylated insulin receptor to interact with and phosphorylate IRS molecules *(23,30,31)*. In addition, these insulin receptor substrates contain multiple phosphotyrosine motifs that can bind to SH2 domains and may serve as docking molecules that mediate the formation of signaling complexes consisting of several SH2 domain–containing proteins.

Signaling Proteins Containing SH2 and SH3 Domains

Many of the signaling molecules participating in RTK signal transduction pathways contain SH2 and/or SH3 domains that mediate protein-protein interactions. As mentioned previously, SH2 domains interact specifically with phosphorylated tyrosine motifs. SH3 domains bind with high affinity to particular proline-rich sequences *(4)*. Some SH2 domain-containing proteins (e.g., SHP-2, PLC-γ) are effector molecules that possess intrinsic catalytic activity that is regulated or localized by interactions of the SH2 domain of the effectors with phosphotyrosine motifs on other proteins (e.g., IRS-1). Other SH2/SH3 domain-containing proteins (e.g., GRB-2, Nck, and the p85 regulatory subunit of PI3K) are known as adaptor proteins because they have no intrinsic catalytic activity and their function involves forming specific signaling complexes mediated by the simultaneous interactions of multiple SH2/SH3 domains on the adaptor protein with both upstream and downstream signaling molecules. Activation of Ras and PI3K, two major effector pathways common to a number of growth factor receptors including the insulin receptor, fit this latter pattern. For example, GRB-2 is normally prebound to SOS (a guanine nucleotide exchange factor) via interactions of the two SH3 domains of GRB-2 and proline-rich regions of SOS. When phosphotyrosine motifs on IRS-1 and Shc interact with the SH2 domain of GRB-2, activation of the prebound SOS promotes formation of the GTP-bound form of Ras, leading to activation of Ras. Similarly, the p85 regulatory subunit of PI3K is normally preassociated with the p110

catalytic subunit. Insulin stimulation results in the interaction of phosphotyrosine motifs on IRS proteins with SH2 domains on p85, leading to activation of the prebound p110 catalytic subunit (for reviews see refs. *32* and *33*).

Downstream Phosphorylation Cascades

Distal RTK signaling pathways are difficult to dissect cleanly because multiple branching pathways begin to emerge from single effectors. Adding to the complexity, multiple upstream inputs often converge on single branch points. Furthermore, negative feedback mechanisms sometimes exist that lead to downstream signals affecting upstream components. However, it is clear that various serine/threonine phosphorylation cascades contribute to the propagation of signaling from the cell surface to the nucleus. These phosphorylation cascades seem to be common to signaling for many growth factors including insulin. For example, Ras directly activates Raf, a serine/threonine kinase that phosphorylates and activates MEK, which in turn phosphorylates and activates mitogen-activated protein kinase (MAPK), leading to induction and activation of early immediate genes such as the protooncogenes c-*jun* and c-*fos*. Insulin signaling mediated by PI3K pathways also involves downstream serine/threonine kinase cascades. For example, phospholipid products generated by PI3K activate PDK1, a serine/threonine kinase that phosphorylates and activates Akt (another serine/threonine kinase), which in turn phosphorylates and inactivates glycogen synthase kinase-3 (GSK-3) *(34–36)*. This process results in activation of glycogen synthase and the stimulation of glycogen synthesis.

Protein Tyrosine Phosphatases

Since tyrosine phosphorylation is critical to initiating and propagating signaling by RTKs, it is not surprising that dephosphorylation of tyrosine residues by protein tyrosine phosphatases (PTPases) contributes to the regulation of signaling. The number and diversity of PTPases rivals that of the RTKs *(37,38)*. PTPases are generally subdivided into a family of nontransmembrane proteins containing a single catalytic PTPase domain and a family of transmembrane receptor-like PTPases that typically contain tandem PTPase domains. The transmembrane PTPases (also known as receptor-like PTPases) have been further categorized into eight groups based on shared structural features of various extracellular domains *(38)*. The large number of PTPases discovered and characterized to date suggests that each PTPase plays a specific role in modulating signaling by RTKs. PTPases such as SHP-2 contain SH2 domains that confer specificity whereas the receptor-like PTPases have extracellular domains that presumably interact with specific ligands. In addition, subcellular localization of particular PTPases may contribute to their specificity. Although all of the determinants of PTPase specificity are not understood, there is evidence that particular PTPases show selectivity for specific RTKs *(39)*.

In the case of signaling by the insulin receptor, the transmembrane PTPases, PTP-α, PTP-ε, and LAR, have all been implicated as modulators of insulin action *(40–42)*. In particular, LAR has been shown to interact with and dephosphorylate the insulin receptor in intact cells *(43)*. In addition, the expression and level of activity of LAR in insulin targets such as muscle and adipose tissue is increased in insulin-resistant states such as obesity and diabetes *(44,45)*. Among the nontransmembrane PTPases, PTP1B and SHP-2 have both been shown to modulate insulin signaling. PTP1B dephos-

phorylates the insulin receptor both in vitro and in intact cells *(39,46,47)*. In addition, PTP1B regulates both mitogenic and metabolic actions of insulin *(41,48,49)*. In tissue culture models, an increase in the level and activity of PTP1B has been associated with insulin resistance induced by exposure to high glucose levels. In addition, the level and activity of PTP1B in human skeletal muscle is positively correlated with in vivo measures of insulin sensitivity *(50–52)*. Binding of the SH2 domains of SHP-2 to phosphotyrosine motifs on either the insulin receptor or IRS-1 results in activation of SHP-2 PTPase activity *(53,54)*. Interestingly, a number of studies have shown that SHP-2 participates in Ras- and MAPK-dependent pathways as a positive mediator of mitogenic actions of insulin and other growth factors *(55–58)*.

INSULIN SIGNALING PATHWAYS THAT REGULATE GLUCOSE TRANSPORT

A primary metabolic function of insulin that distinguishes it from other growth factors is the promotion of whole-body glucose utilization and disposal. The rate-limiting step in glucose utilization under normal conditions is glucose transport into cells. The insulin-responsive glucose transporter GLUT4 is expressed at high levels almost exclusively in classical insulin targets such as muscle and adipose tissue (for a review see ref. *59*). Insulin stimulates increased glucose transport in these tissues by causing the redistribution of GLUT4 from an intracellular pool to the cell surface, where it acts as a facilitative transporter to enhance entry of glucose into the cell *(60–62)*. This redistribution of GLUT4 is due largely to insulin increasing the rate of exocytosis of GLUT4 (insulin may also have a minor effect in decreasing endocytosis of GLUT4) *(63–65)*.

Although the tissue-specific distribution of GLUT4 and the effects of insulin on the subcellular localization of GLUT4 have been known for some time, elucidation of metabolic insulin signaling pathways has lagged behind other areas of insulin signal transduction for several reasons. First, although muscle and adipose tissue normally express high levels of GLUT4 and are extremely responsive to insulin stimulation, the ability to apply modern molecular methods such as transfection of recombinant DNA to these terminally differentiated tissues has been limited. Second, tissue culture models of muscle and adipose cells that are easier to manipulate (e.g., 3T3-L1 adipocytes, L6 myocytes, or C2C12 cells) do not always faithfully reflect important characteristics of bona fide insulin target cells. For example, the relative levels of expression of IRS-1, -2, and -3 are quite different in primary adipose cells and 3T3-L1 adipocytes *(25,66,67)*. Third, the requisite cellular machinery for appropriate subcellular trafficking of GLUT4 seems to be lacking in commonly used tissue culture cells such as NIH-3T3 fibroblasts, Chinese hamster ovary cells, or COS cells, which do not normally express GLUT4. Therefore, even when recombinant insulin receptors and GLUT4 are stably expressed in these cells, they are much less responsive to insulin than muscle or adipose cells *(68)*.

The recent use of electroporation to transfect adipose cells in primary culture in conjunction with quantitative methods for assessment of cell surface GLUT4 has led to a clearer understanding of metabolic insulin signaling pathways *(41,69–75)*. In addition, transgenic mice that have had key signaling molecules either knocked out or overexpressed have provided valuable insights *(76–80)*. Finally, microinjection or viral

transfection strategies in differentiated 3T3-L1 adipocytes along with semiquantitative methods for assessing cell surface GLUT4 have also been informative *(81–85)*. Figure 2 summarizes some of what is currently known about insulin signaling pathways related to the translocation of GLUT4 in adipose cells.

Since insulin receptor autophosphorylation and enhancement of RTK activity are among the earliest known events in insulin signaling, one might predict that RTK activity is necessary for most, if not all, biological actions of insulin including metabolic actions such as recruitment of GLUT4 to the cell surface. This idea was supported by the identification of kinase-deficient insulin receptor mutants in some patients with syndromes of extreme insulin resistance *(86)*. Direct evidence that insulin RTK activity is important for mediating the effect of insulin to stimulate translocation of GLUT4 in insulin target cells has been obtained using transfected rat adipose cells in primary culture (Fig. 3) *(70)*. Cells overexpressing wild-type insulin receptors showed a marked increase in cell surface GLUT4 in the absence of insulin when compared with control cells transfected with an empty expression vector. In contrast, cells overexpressing a kinase-deficient mutant insulin receptor had an insulin dose-response curve similar to that of the control cells. Taken together, these data suggest that intact RTK activity is necessary to mediate signaling from the insulin receptor to translocation of GLUT4. Furthermore, it is likely that unoccupied insulin receptors have a low level of intrinsic tyrosine kinase activity whose signal is proportional to the amount of receptors expressed. Additional evidence that the insulin RTK is important in metabolic signaling comes from studies on PTPases such as LAR and PTP1B that are known to dephosphorylate the insulin receptor. Both of these PTPases have been implicated in the negative regulation of metabolic signaling by insulin *(39,41,43,45–47,49)*. In particular, overexpression of PTP1B in rat adipose cells leads to a significant decrease in the level of GLUT4 at the cell surface in both the absense and presence of insulin *(41)*. The fact that PTP1B decreases cell surface GLUT4 in the absence of insulin provides further support for the idea that a small signal is generated by the intrinsic tyrosine kinase activity of unoccupied receptors.

Downstream from the insulin RTK, a number of insulin receptor substrates play roles in insulin-stimulated translocation of GLUT4. Overexpression of IRS-1 in transfected rat adipose cells leads to an increase in cell-surface GLUT4 in the absence of insulin similar to that seen with overexpression of the insulin receptor *(71)*. Interestingly, transfection of adipose cells with an antisense ribozyme against IRS-1 results in a decrease in insulin sensitivity without a decrease in maximal responsiveness with respect to translocation of GLUT4 *(71)*. Thus, although IRS-1 is capable of mediating the effect of insulin to stimulate translocation of GLUT4, other parallel pathways are probably involved. Indeed, the fact that transgenic IRS-1 knockout mice are only mildly insulin resistant provides unequivocal evidence that IRS-1 contributes to metabolic actions of insulin but is not absolutely required for insulin-stimulated glucose uptake *(76,77)*. Overexpression of IRS-2 *(74)* and IRS-3 *(143)* in rat adipose cells also leads to translocation of GLUT4 in the absence of insulin, suggesting that these substrates may also contribute to metabolic actions of insulin. Of note, in adipose cells, the time course for the association of IRS-3 with the p85 regulatory subunit of PI3K in response to insulin stimulation is much more rapid than for IRS-1 *(87)*. In addition, the magnitude of the association between IRS-3 and p85 in response to insulin seems to be greater than for IRS-1. Furthermore, in transgenic mice lacking IRS-1, IRS-3 is the insulin

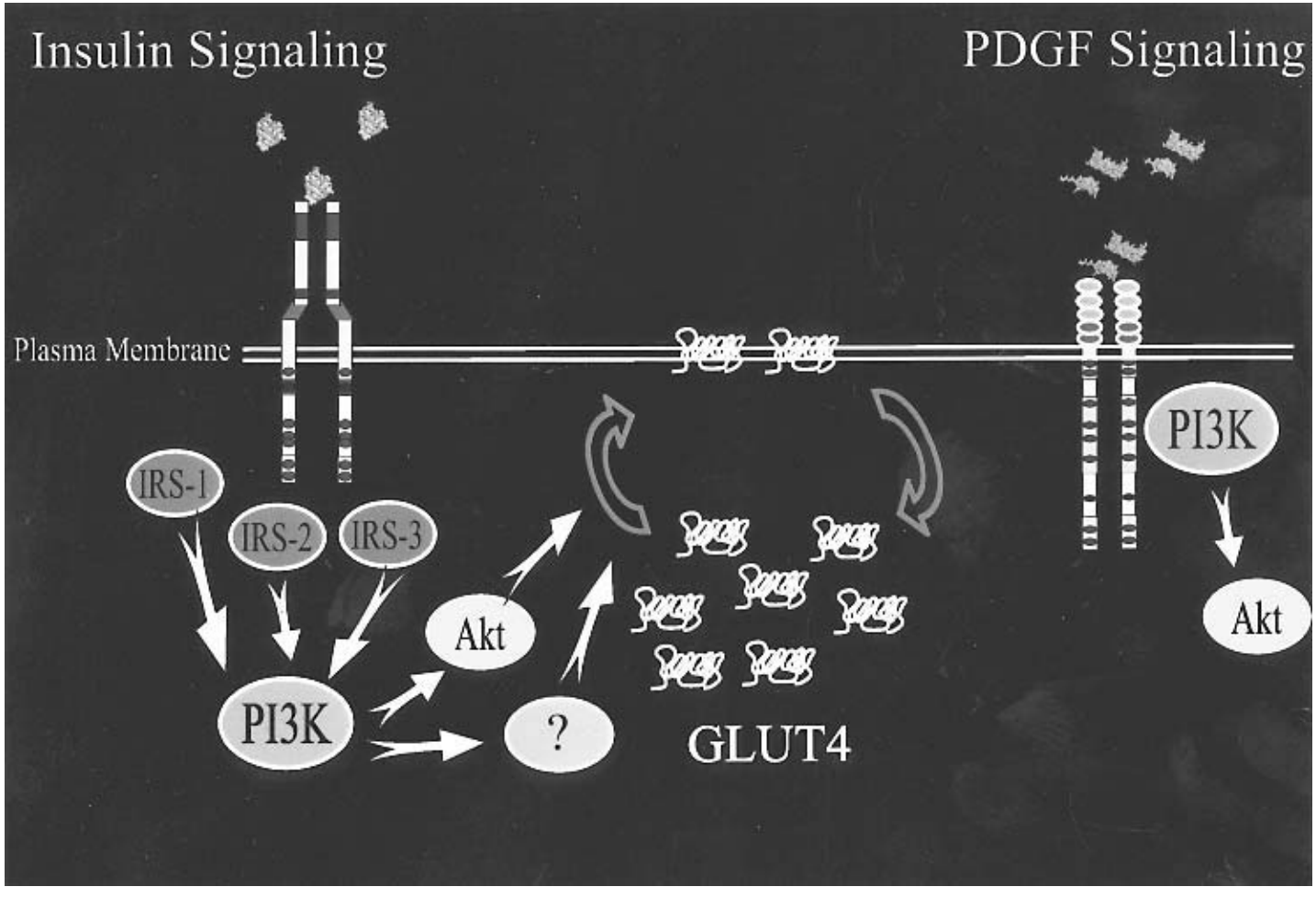

Fig. 2. Insulin signaling pathways that contribute to translocation of GLUT4 in adipose cells. Interestingly, although activation of PI3K is necessary for insulin-stimulated translocation of GLUT4, it does not appear to be sufficient because activation of PI3K by PDGF is without effect on translocation of GLUT4 when PDGF receptors are expressed at physiological levels.

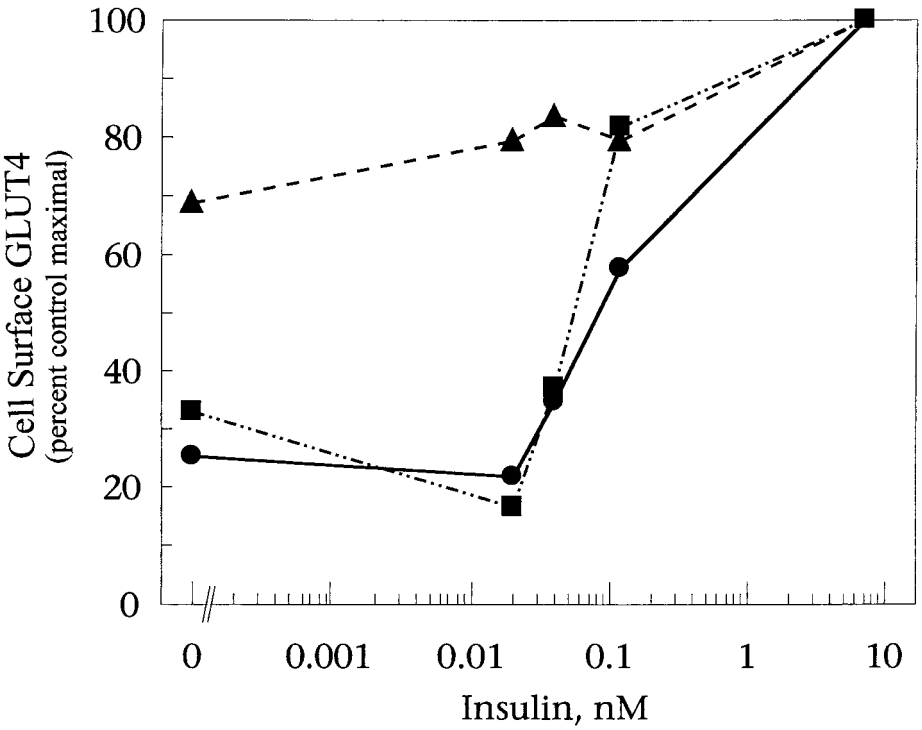

Fig. 3. Insulin RTK activity is important for insulin-stimulated translocation of GLUT4. Rat adipose cells were cotransfected with an epitope-tagged GLUT4 and wild-type human insulin receptors (▲), tyrosine kinase–deficient mutant insulin receptors cells (■), or an empty expression vector (control) (●). Cell-surface concentrations of epitope-tagged GLUT4 are shown as a function of insulin concentration (expressed as a percentage of the maximally stimulated control cells) *(70)*.

receptor substrate in adipose cells responsible for the majority of activation of PI3K in response to insulin *(87,88)*. Since PI3K is necessary for insulin-stimulated glucose transport (*see* the next paragraph), these data suggest that IRS-3 may be a major insulin receptor substrate mediating metabolic actions in vivo.

As already mentioned, two major insulin signaling pathways downstream from the receptor substrates are the PI3K- and the Ras-dependent pathways. Overexpression of constitutively active mutants of either PI3K or Ras in adipose cells leads to massive recruitment of GLUT4 to the cell surface in the absence of insulin *(72,89)*. However, overexpression of recombinant proteins can sometimes lead to effects that do not occur under physiological conditions. Interestingly, when dominant inhibitory mutants were used to knock out either endogenous PI3K or Ras in adipose cells, overexpression of the PI3K mutant resulted in a nearly complete inhibition of insulin-stimulated translocation of GLUT4 (Fig. 4) whereas overexpression of the Ras mutant did not cause a significant change in the insulin dose-response curve *(72)*. Thus, even though constitutively active PI3K and Ras are both capable of stimulating the recruitment of GLUT4 to the cell surface, it appears that only PI3K plays a necessary physiological role in this process. However, PI3K activity per se is not sufficient to cause translocation of GLUT4 because stimulation of PI3K activity in adipose cells using other growth factors such as PDGF does not result in translocation of GLUT4 *(73,90)*.

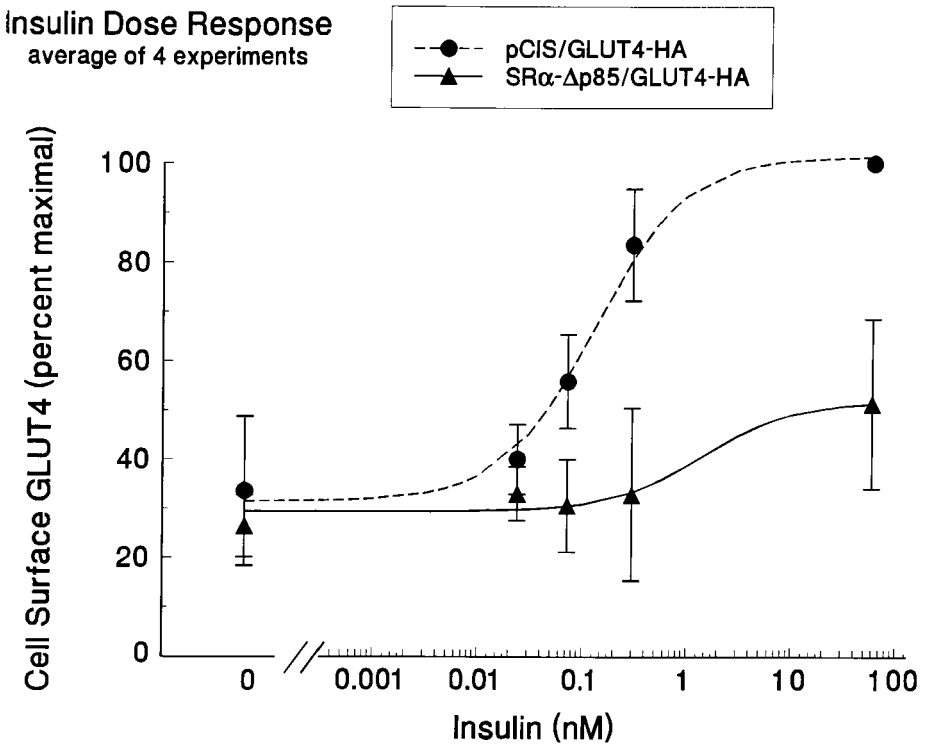

Fig. 4. PI3K is necessary for insulin-stimulated translocation of GLUT4. Rat adipose cells were cotransfected with an epitope-tagged GLUT4 and either a dominant inhibitory mutant of the p85 regulatory subunit of PI3K (▲) or an empty expression vector (control) (●). Cell-surface concentrations of epitope-tagged GLUT4 are shown as a function of insulin concentration (expressed as a percentage of the maximally stimulated control cells) *(72)*. Data represent insulin dose response for an average of four experiments.

There are several effectors downstream of PI3K that may play a role in insulin-stimulated translocation of GLUT4. Akt is a serine/threonine kinase that is activated by insulin via lipid products of PI3K binding to the PH domain of Akt, and phosphorylation of critical serine and threonine residues on Akt by phosphoinositide-dependent kinase-1, another kinase downstream of PI3K that is activated by lipid products of PI3K *(34,91,92)*. Like PI3K and Ras, overexpression of constitutively active mutants of Akt in rat adipose cells or 3T3-L1 adipocytes leads to massive recruitment of GLUT4 to the cell surface *(75,84)*. However, in contrast to PI3K, dominant inhibitory mutants of Akt that are kinase deficient only partially inhibit insulin-stimulated translocation of GLUT4 in adipose cells (Fig. 5). This suggests the possibility that multiple downstream effectors of PI3K contribute to mediating the translocation of GLUT4. For example, the atypical PKC isoform PKC-ζ is a good candidate for another downstream effector of PI3K that may contribute to metabolic signaling by insulin. In 3T3-L1 adipocytes, overexpression of a constitutively active PKC-ζ mutant increased glucose transport whereas overexpression of a dominant inhibitory PKC-ζ mutant decreased insulin-stimulated glucose transport *(93)*.

In addition to the progress being made by tracing signaling pathways starting from

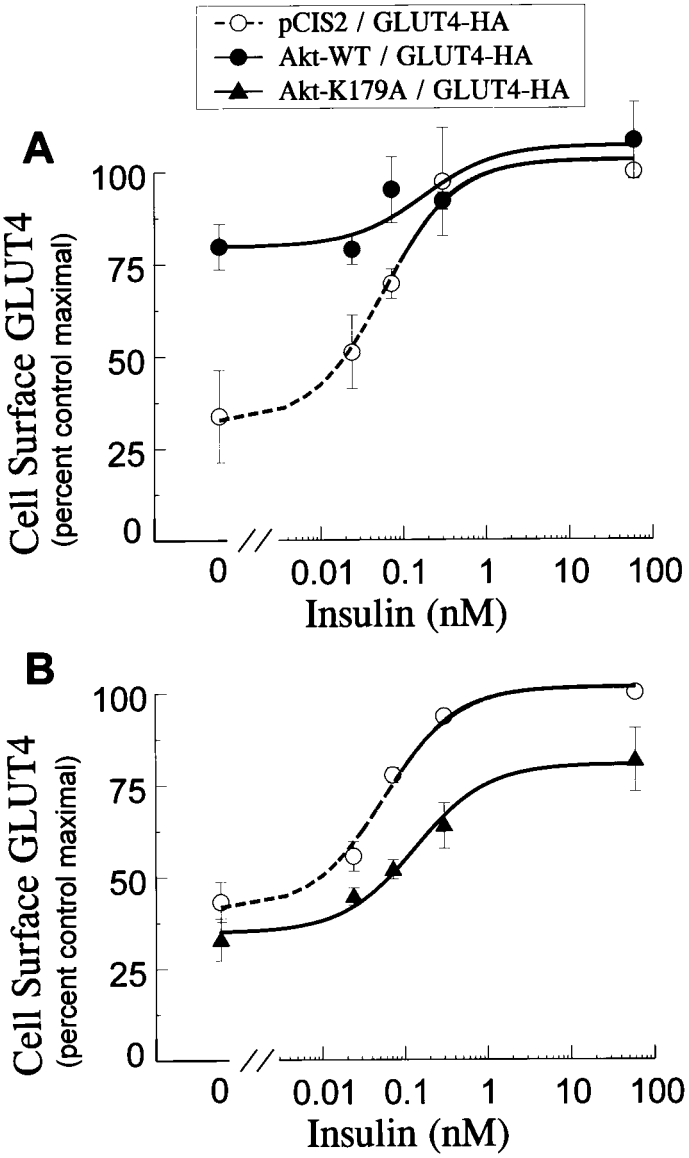

Fig. 5. Physiological role for Akt in insulin-stimulated translocation of GLUT4. Rat adipose cells were cotransfected with an epitope-tagged GLUT4 and wild-type Akt (●), a kinase inactive mutant Akt (▲), or an empty expression vector (control) (○). Cell-surface concentrations of epitope-tagged GLUT4 are shown as a function of insulin concentration (expressed as a percentage of the maximally stimulated control cells) *(75)*.

the insulin receptor, progress has also been made in understanding the molecular mechanisms underlying the subcellular trafficking of GLUT4 from an intracellular compartment to the cell surface in response to insulin. Mechanisms common to vesicular trafficking during regulated exocytosis of synaptic vesicles in neurons also apply to the subcellular localization of GLUT4. In general, vesicle docking and fusion to the plasma membrane is mediated by specific interactions of soluble *N*-ethylmaleimide-

sensitive factor attachment protein receptors (SNAREs) *(94)*. v-SNARE proteins are localized to the vesicle membrane and t-SNARE proteins are localized to the target plasma membrane. There is good evidence that specific isoforms of v-SNARE and t-SNARE molecules are involved with GLUT4 trafficking (for a review see ref. *95*). For example, VAMP2 (a v-SNARE) is localized to GLUT4-containing vesicles in adipose cells and appears to participate in insulin-stimulated exocytosis of GLUT4 *(96–98)*. Syntaxin 4 (a t-SNARE) binds specifically to VAMP2 and is localized to the plasma membrane in muscle and adipose cells. Furthermore, insulin-stimulated translocation of GLUT4 in 3T3-L1 adipocytes can be blocked by using antibodies against Syntaxin 4 or overexpressing the cytoplasmic tail of Syntaxin 4 *(98–100)*. Presumably, the signaling pathways leading from the insulin receptor interface at some point with the vesicular trafficking machinery for GLUT4. An important goal of current investigations related to metabolic actions of insulin is to identify and characterize direct interactions between signaling proteins and trafficking machinery.

MECHANISMS FOR ACHIEVING SPECIFICITY

Although insulin signaling follows a general paradigm for signaling by RTKs and many downstream signaling components are shared in common with other RTK signaling pathways, the biological actions resulting from insulin stimulation such as increased glucose transport are quite specific and distinctive. At each step in the signal transduction pathway, there are opportunities and potential mechanisms for incorporating signal specificity. Here we briefly discuss selected examples that illustrate potential mechanisms that may be used to achieve specificity in insulin signaling.

Specificity at the Receptor Level

The binding affinity between insulin and its receptor is quite high and provides an obvious first determinant of signal specificity. However, insulin is also capable of binding and activating other related receptors such as the insulin-like growth factor-1 (IGF-1) receptor. Similarly, IGF-1 is capable of binding and activating the insulin receptor *(101)*. Furthermore, because the insulin receptor and IGF-1 receptor are homologous, the formation of hybrid receptors with an insulin receptor αβ-subunit joined to an IGF-1 αβ-subunit can occur. These hybrid receptors are capable of undergoing transphosphorylation and may generate unique signals. Since the relative amounts of insulin receptors and IGF-1 receptors differ in particular tissues, the numbers of pure receptors and hybrid receptors may vary from tissue to tissue. Although the binding affinities of insulin and IGF-1 for the heterologous receptor are approx 100-fold less than for their own receptor, the integration of multiple signals at different amplitudes may contribute to the determination of specific effects. For example, in vascular endothelial cells that normally express 10 times as many IGF-1 receptors as insulin receptors, stimulation with insulin at concentrations sufficient to saturate both IGF-1 and insulin receptors results in the production of nitric oxide at a level twice that seen with stimulation by IGF-1 at comparable concentrations *(102)*. Additional evidence that the binding interaction between ligand and receptor affects signaling specificity comes from studies with point mutants of insulin molecules that have been designed to have higher binding affinities for the insulin receptor than the native insulin molecule. For

example, the Asp B10 insulin mutant has a much higher binding affinity than native insulin for the insulin receptor and appears to favor mitogenic rather than metabolic actions of insulin *(103)*. Another feature of insulin binding to its receptor that may affect signal specificity is the fact that insulin binding exhibits negative cooperativity *(104)*. That is, the binding affinity of insulin for its receptor decreases with increasing insulin concentrations. Thus, the dynamics of intracellular signaling events in response to a particular insulin secretory profile may encode some specificity. Finally, integration of signals generated by cross talk between different types of receptors may contribute to the specificity of insulin signaling. For example, in addition to the well-known cross talk that occurs between insulin and IGF-1 at the receptor level, there is evidence for cross talk between insulin and PDGF signaling with respect to interactions between IRS-1 and PI3K *(105)*. Furthermore, recent evidence suggests that activation of G-protein-coupled receptors such as the angiotensin II receptor can influence insulin signaling through interactions with IRS-1 and -2 *(106–108)*.

Specificity at the Receptor Substrate Level

The existence of multiple substrates of the insulin receptor also provides opportunities to incorporate specificity. Members of the IRS family of substrates contain multiple phosphotyrosine docking sites for SH2 domain–containing proteins. The number of these docking sites and the particular SH2 domains with which they interact vary among the different IRS proteins. That is, the combination of downstream signaling molecules engaged by an IRS protein as well as the relative affinities of particular downstream effectors for each substrate are unique for each IRS protein. Thus, tissue-specific differences in the relative expression levels of these IRS substrates may result in formation of distinct signalling complexes in particular tissues and help explain why some actions of insulin predominate in certain tissues *(66,109)*. In addition, in some downstream signaling molecules containing tandem SH2 domains (e.g., SHP-2 and the p85 regulatory subunit of PI3K), the spatial relationship between these SH2 domains provides an additional level of specificity. That is, the geometry of multiple phosphotyrosine motifs on a particular substrate is important for optimal binding and activation of proteins with tandem SH2 domains *(110,111)*. Similarly, the relationship of SH2 and SH3 domains in various adaptor or effector molecules may impose physical constraints on the formation of signaling complexes that are important for signal specificity.

In addition to members of the IRS family, there are other substrates of the insulin receptor that are also expressed in a tissue-specific manner and may contribute to specificity in insulin signaling. For example, there is a family of M_r 120,000 integral membrane glycoproteins that are phosphorylated by the insulin receptor. pp120/HA4 was the first member of this family to be identified as a substrate for the insulin receptor *(112–116)*. Based on the sequence flanking the tyrosine phosphorylation site in pp120/HA4, Najjar et al. *(117)* predicted that the protein would bind to the SH2 domain of SH2-containing phosphotyrosine phosphatases. Subsequently, two other laboratories identified two homologous glycoproteins (SHP substrate-1 *[118]* and signal-regulatory protein [SIRP] *[119]*) that were phosphorylated by the insulin receptor and other tyrosine kinases. Furthermore, the phosphorylated proteins did indeed bind to SHP-1 and SHP-2, and served as substrates for these two phosphotyrosine phosphatases. Moreover, SIRP was demonstrated to exert an inhibitory effect on signaling through RTKs.

Subcellular Compartmentalization of Signaling Complexes

Signal specificity may also be determined by localization of signaling complexes to particular subcellular compartments. For example, in adipose cells, there is evidence that insulin stimulation results in the localization of IRS-1/PI3K complexes to GLUT4-containing vesicles *(120)*. The subcellular targeting of PI3K by insulin may help explain why activation of PI3K by insulin results in translocation of GLUT4 but similar activation of PI3K by PDGF does not *(73,90)*. The fact that PDGF stimulation of adipose cells overexpressing PDGF receptors results in translocation of GLUT4 is consistent with the idea that overexpression of proteins may lead to aberrant localization of signaling molecules in compartments where they would normally be excluded *(73)*.

Another subcellular compartment that may contribute importantly to organizing microdomains of signaling complexes are caveolae (small invaginations in the plasma membrane that contain scaffold-like proteins such as caveolins) *(121,122)*. Caveolae are quite abundant in terminally differentiated cell types such as muscle, endothelial, and adipose cells. Furthermore, growth factor receptors such as PDGF and EGF as well as other signaling proteins such as Ras, MAPK, phosphoinositides, G-proteins, calmodulin, and nitric oxide synthase have all been localized to caveolae (some of these interact directly with caveolin) *(123–126)*. Recently it was shown that all the necessary factors for PDGF-stimulated MAP kinase activation (including the PDGF receptor, Ras, Raf1, Mek1, and Erk2) are localized and functionally active in caveolae *(123)*. Interestingly, in 3T3-L1 cells, insulin stimulates the phosphorylation of caveolin only when the cells are differentiated into adipocytes, but not in the fibroblast form *(127)*. Furthermore, in endothelial cells, the interaction of caveolin with nitric oxide synthase is modulated by tyrosine phosphorylation *(128,129)*. Therefore, it is conceivable that some of the specificity in insulin signaling is determined by the organization of signaling complexes in caveolae or other similar subcellular compartments.

Tissue-Specific Expression of Key Effectors

Specific biological responses to insulin may also be determined, in part, by tissue-specific expression of signaling and effector molecules that are necessary for particular actions of insulin. For example, in the case of insulin-stimulated glucose transport, GLUT4 is the major insulin-responsive glucose transporter that is recruited to the cell surface in response to insulin. Since GLUT4 is predominantly expressed in skeletal muscle and adipose tissue, the effect of insulin to increase glucose transport occurs mostly in these tissues. However, transfecting other cell types (e.g., fibroblasts) with GLUT4 and insulin receptors is not sufficient to make cells as responsive to insulin with respect to glucose transport as classical insulin target cells. Thus, there are presumably additional tissue-specific signaling elements important for insulin-stimulated glucose transport besides the insulin receptor and GLUT4.

Another example of the importance of tissue-specific expression of key effectors is demonstrated by the recent finding that caveolin is tyrosine phosphorylated in response to insulin stimulation only in differentiated 3T3-L1 adipocytes, not in undifferentiated 3T3-L1 fibroblasts *(127)*. The phosphorylation of caveolin can be mediated by the kinase fyn, which is thought to be activated by the binding of phosphorylated c-*cbl* in response to insulin stimulation. Interestingly, although the insulin receptor, c-*cbl*, fyn,

and caveolin are all expressed in both 3T3-L1 fibroblasts and adipocytes, insulin stimulation results in phosphorylation of c-*cbl* only in the differentiated 3T3-L1 adipocyte *(130)*. This implies that the kinase responsible for phosphorylation of c-*cbl* in response to insulin (or some other upstream component) is expressed only in the adipocyte, not the fibroblast form of 3T3-L1 cells, and may explain why caveolin is phosphorylated in response to insulin only in adipocytes.

The existence of multiple isoforms of key signaling molecules may also be important for signal specificity. For example, PI3K is essential for insulin-stimulated glucose transport. However, multiple isoforms and splice variants of both the regulatory p85 and catalytic p110 subunits of PI3K that have differential responses to insulin have been discovered *(131–137)*. Each of these isoforms may generate a distinct pattern of lipid products that have specific roles in signaling. The lipid products of PI3K are known to bind to PH domains of downstream effectors, resulting in activation or regulation of these PH domain–containing molecules. Recently, different lipid products of PI3K were shown to have differential binding affinities for particular PH domains from various signaling molecules *(138)*. Thus, the combination of different isoforms of regulatory and catalytic subunits of PI3K in conjunction with tissue-specific expression and localization to subcellular compartments may result in the generation of a particular profile of lipid products that interact in specific ways with downstream effectors that determines the biological response to insulin stimulation.

Feedback Regulation

The function of end products to dampen signals from one pathway while amplifying signals from others is a common mechanism used in the regulation of enzymatic pathways. It is possible that specificity in RTK signal transduction is also determined, in part, by positive or negative feedback. In the case of insulin signaling, it was recently shown that GSK-3 (a downstream metabolic effector of insulin inactivated by Akt) can phosphorylate IRS-1 on serine/threonine residues and inhibit insulin RTK activity *(139)*. Similarly, PI3K (downstream from IRS-1) has serine/threonine kinase activity in addition to its lipid kinase activity and phosphorylates IRS-1 on serine residues, which may result in modulation of IRS-1 function *(140)*. In addition, there is evidence that PI3K has functional interactions both upstream and downstream from Ras, suggesting another feedback loop that may be involved with insulin signaling *(141,142)*.

Modulation of Signal Frequency and Amplitude

Cellular signals generated by changes in ion fluxes or membrane potential often encode information in the modulation of the signal frequency and amplitude. It is conceivable that the dynamics of signaling by RTKs also encode specific information by modulation of the frequency and amplitude of various phosphorylation cascades. For example, it was recently shown that the time course for association between PI3K and IRS-3 in rat adipose cells in response to insulin stimulation is faster than for IRS-1 *(87)*. Furthermore, in the same study, the amount of PI3K associated with IRS-3 in response to insulin stimulation was also greater than for IRS-1. This difference in the time course and amplitude of PI3K activation may help distinguish signals that are mediated by IRS-1 from those by IRS-3 and ultimately result in different biological effects.

SUMMARY

The molecular mechanisms of insulin action follow a general paradigm for RTK signal transduction. As a result, significant progress has been made in recent years to elucidate the insulin signaling pathways involved with the promotion of glucose uptake and metabolism, one of the most distinctive and important biological actions of insulin. A fundamental challenge for future investigations is to understand how specific biological actions of insulin are determined using signaling molecules that are common to signaling pathways used by many other growth factors and cytokines. Convergence and divergence of multiple branching pathways, subcellular compartmentalization, tissue-specific expression of key effectors, and modulation of signal frequency and amplitude are among the potential mechanisms underlying specificity in insulin signaling.

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To date, no AF-1 specific coactivator has been isolated. This will clearly be of great interest, because partial agonists of estrogen and progesterone are believed to exert their actions through this domain. SRC-1 has been reported to interact with both the AF-1 and AF-2 domains, and may contribute to the AF-1 effect in at least some cases *(94)*. Thus, there may be coactivator or modulator proteins that interact with AF-2, AF-1, or both domains. A novel hinge domain–binding coactivator, L7/SPA, has been isolated from HeLa cells, and increased the partial agonist activity of TAM-bound ER and RU486-occupied PR *(76)*.

In general, unliganded ER does not bind to corepressors such as silencing mediator of retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (NCoR), which bind to the hinge region of nuclear receptors, such as the thyroid hormone receptors and RARs, and prevent binding of the LBD regions to coactivators *(41)*. In these receptors, the corepressor proteins invoke a receptor conformation that actively represses transcription, owing partially to histone acetylase activity of the coactivator proteins. Specific conformations of ER, resulting from binding to receptor antagonists, may result in corepressor recruitment to the liganded receptor complexes. For example, in HepG2 liver cells, in which tamoxifen is a partial agonist, exogenous SRC-1 enhanced E and TAM-stimulated transcription, whereas overexpression of the corepressor SMRT strongly reduced basal and TAM-mediated transcription with no effects on E activity *(86)*. Similarly, PR bound to antiprogestins of the partial agonist class binds more effectively to corepressors N-CoR and SMRT than does PR bound to other ligands *(76,95)*, and this association can be suppressed by treatment of cells with cAMP *(95)*. Unliganded PR may bind to corepressors, and additional corepressors with more complicated or specific receptor requirements may exist. For example, human ER LBD bound to antiestrogens such as tamoxifen, but not bound to E, associates with at least one nuclear protein capable of acting as a corepressor *(96)*. Such molecules would not be isolated using only ligand-bound receptors as bait in typical two-hybrid or other protein interaction assays. Additional studies will undoubtedly focus on the types of accessory proteins bound to specific receptors with various ligands, as well as modifications of those proteins by intracellular signaling cascades.

SUMMARY

Overall, several factors including the character of the ligand, the steroid receptor isoform expressed in a specific cell type, and intracellular signaling pathways activated in a given cell or tissue may all be important in determining the character of partial steroid antagonists. At least some of these responses are directly related to the complement of coactivators and corepressors associated with ligand-bound receptor within a given context. As we have discussed, the levels of individual coactivators and corepressors may be modulated physiologically, and it is likely that posttranslational modifications will also occur in response to signaling cascades by growth factors and other bioactive peptides. Individual ligand binding to specific receptor isoforms confers distinct conformational changes and contours to the receptors, capable of interacting with the cellular accessory proteins. Based on both the identity and levels of coactivators and corepressors, the resulting receptor protein complex will have either a stimulatory or suppressive conformation and a resulting effect on model gene transcription. An additional layer of diversity will then be provided by the specific ERE or responsive promoter region

in the cellular target genes, since the receptor conformation may be altered as it binds to different DNA sequences, or contacts different proteins at nearby promoter regions. These interactions can alter the essential character of a given ligand, from antagonist to agonist or the reverse. Current and future studies will be focused on the essential mechanisms underlying such diversity and specificity, and how these processes can be regulated or manipulated for a given positive biological outcome.

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